Nonsteroidal anti-inflammatory drugs are a commonly used treatment for pain and inflammation in horses undergoing surgery. These drugs exert their effects mostly by inhibiting COX activity and subsequent tissue prostanoid production. Two main COX isoforms have been identified: COX-1 is the constitutive form and plays a role in the regulation of tissue blood flow in physiologic states; COX-2 is mainly expressed during inflammatory states, and its uncontrolled action is considered responsible for many of the undesired effects of the inflammatory process.1 In horses, the use of nonselective COX inhibitors, including phenylbutazone and flunixin meglumine, has been associated with serious gastrointestinal and renal toxicoses.2–5 These adverse effects are likely the result of reduced mucosal and renal medullary perfusion as a consequence of reduced COX-1 activity.6 Selective COX-2 inhibitors known as coxibs have been evaluated in horses.7–11 Analysis of the results of a study12 of the recovery for ischemic-injured jejunum in horses suggested that selective inhibitors of the COX-2 isoform might have the potential for fewer adverse effects by inhibiting COX-2 activity but preserving the physiologic activity of the COX-1 isoenzyme, which would allow physiologically normal tissue function. Studies conducted to assess the clinical efficacy of firocoxib have revealed a positive effect on orthopedic pain13,14 comparable to that of nonselective COX inhibitors.9 Currently, firocoxib is the only COX-2 selective drug licensed for use in horses to alleviate pain and inflammation. However, although use of firocoxib

Effect of flunixin meglumine and firocoxib on ex vivo cyclooxygenase activity in horses undergoing elective surgery

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OBJECTIVE
To evaluate ex vivo cyclooxygenase (COX) inhibition and compare in vitro and ex vivo COX-1 inhibition by flunixin meglumine and firocoxib in horses.

ANIMALS
4 healthy horses for in vitro experiments and 12 healthy horses (6 males and 6 females; 5 Thoroughbreds, 5 Warmbloods, and 2 ponies) undergoing elective surgery for ex vivo experiments.

PROCEDURES
12 horses received flunixin meglumine (1.1 mg/kg, IV, q 12 h) or firocoxib (0.09 mg/kg, IV, q 24 h). Blood samples were collected before (baseline) and 2 and 24 hours after NSAID administration. Prostanoids (thromboxane B₂, prostaglandin E₂, and prostaglandin E metabolites) served as indicators of COX activity, and serum drug concentrations were measured by use of high-performance liquid chromatography. An in vitro coagulation-induced thromboxane B₂ assay was used to calculate drug concentration–COX-1 inhibition curves. Effect of time and treatment on COX activity was determined. Agreement between in vitro and ex vivo measurement of COX activity was assessed with Bland-Altman analysis.

RESULTS
At 2 and 24 hours after NSAID administration, COX-1 activity was reduced, compared with baseline activity, for the flunixin meglumine group only and relative COX-1 activity was significantly greater for the firocoxib group, compared with that for the flunixin meglumine group. There was no significant change in COX-2 activity after surgery for either group. Bland-Altman analysis revealed poor agreement between in vitro and ex vivo measurement of COX-1 activity.

CONCLUSIONS AND CLINICAL RELEVANCE
has been shown to be safe, COX-1 sparing has not been detected, except with an experimental model.\textsuperscript{12}

The COX selectivity of NSAIDs is commonly described by the ratio of the drug concentration required to inhibit each enzyme by a specified amount, often 50%. The COX selectivity of an NSAID may depend on the type of experiments (ie, in vitro or ex vivo).\textsuperscript{19,16} Although an earlier study\textsuperscript{17} that involved the use of purified enzymes in vitro identified a COX selectivity ratio of 1,275 for deracoxib in dogs, a subsequent study\textsuperscript{18} performed on whole blood identified a substantially lower ratio of only 12. Some studies have detected little difference in clinical effects or rate of adverse effects for NSAIDs with different COX selectivity in vitro\textsuperscript{19} and few discrepancies between in vitro and ex vivo inhibition of COX activity.\textsuperscript{16,20} These studies revealed that evaluating results of ex vivo as well as in vitro experiments is of pivotal importance for assessing effects that these drugs have on clinical patients.\textsuperscript{21}

To the authors’ knowledge, studies\textsuperscript{8,22,23} have been conducted to assess the effects of NSAIDs on horses only in vitro or in experimental animals. Therefore, the purpose of the study reported here was to investigate the effects of firocoxib and flunixin meglumine on the activity of COX enzymes in clinical equine patients by use of an ex vivo method and compare results of ex vivo and in vitro measurement of COX-1 activity.

Materials and Methods

ANIMALS

Sixteen horses were included in the study. Four horses were used in the in vitro portion of the study, and 12 client-owned horses were used in the ex vivo portion of the study. All procedures were approved by the Ethics and Welfare Committee of the University of Glasgow.

IN VITRO INHIBITION OF COX-1 ACTIVITY

A convenience population (2 Thoroughbreds, 1 Warmblood, and 1 pony [2 mares and 2 geldings]) from the teaching and blood donation herd of the Weipers Centre Equine Hospital of the University of Glasgow were included in the study. Median age was 20 years (range, 8 to 28 years), and median body weight was 572 kg (range, 296 to 615 kg). None of the horses had a history of systemic disease; all horses were up to date with regard to vaccination status, had no abnormal physical examination findings,\textsuperscript{24} and had a total protein concentration and PCV within respective reference ranges.\textsuperscript{25}

MEASUREMENT OF IN VITRO INHIBITION OF COX-1 ACTIVITY

Effects of firocoxib and flunixin meglumine on in vitro COX-1 activity were determined by measuring coagulation-induced TXB\textsubscript{2} as described elsewhere.\textsuperscript{22,26} Residual blood obtained during routine health monitoring of the 4 horses was used in an in vitro assay. Briefly, whole blood (500 µL) was added to polypropylene microcentrifuge tubes containing firocoxib or flunixin meglumine at a final concentration ranging from 0.01 to 1,000µM (6 dilutions; duplicates for each NSAID at each dilution). A 500-µL sample of heparinized (uncoagulated) blood was collected to serve as a negative control sample. Whole blood (500 µL; in triplicate) was treated with vehicle (positive control sample). Blood was allowed to clot for 1 hour at 37°C and then centrifuged at 2,000 x g for 10 minutes. Then, 100 µL of serum was harvested and added to 400 µL of methanol, and the resulting solution was centrifuged at 6,000 x g for 10 minutes. A 50-µL aliquot of supernatant was collected and diluted in 150 µL of buffer from a commercially available TXB\textsubscript{2} EIA kit; that kit was used to determine the amount of TXB\textsubscript{2} in each sample.

Inhibition of COX-1 activity was calculated as the percentage change in TXB\textsubscript{2} concentration, compared with results for the negative control sample. The percentage change was plotted against the corresponding drug concentration, and the best sigmoid curve fit obtained by use of nonlinear regression was calculated in a statistical software package.\textsuperscript{26} The equation to predict the relative inhibition of COX-1 activity expressed as the change in TXB\textsubscript{2} concentration relative to the concentration of the drug was as follows:

\[
\text{Percentage inhibition} = y_0 + \left(\frac{a}{1 + e^{-\left(x - x_0\right)/b}}\right)
\]

where \(y_0\) is the minimum COX activity, \(a\) is the difference between the maximum and minimum COX activity, \(e\) is the base of the natural logarithm, \(x\) is the concentration of the drug, \(x_0\) is the drug concentration required for 50% inhibition of COX activity, and \(b\) is the slope.

EX VIVO EFFECTS OF FLUNIXIN MEGLUMINE AND FIROCOXIB

Twelve equine patients evaluated at the Weipers Centre Equine Hospital at the University of Glasgow between April and September 2012 were included in the study. Inclusion criteria were a history of no medication within the 2 weeks prior to admission, no abnormalities for physical examination variables (including heart rate, respiratory rate, rectal temperature, mucous membrane color, and capillary refill time),\textsuperscript{24} no abnormalities for hematologic variables,\textsuperscript{25} and an up-to-date vaccination status. Horses < 1 year old were excluded. Horses were included if they were undergoing an elective surgical procedure and sufficient residual blood (2.5 mL) remained from clinical samples obtained for the purposes of hematologic evaluation or serum biochemical analysis prior to surgery, during surgery, and the day after surgery that could be used for measurement of COX activity.

Horses were grouped on the basis of the NSAID selected for use by the attending clinician prior to surgery. Horses received flunixin meglumine (1.1 mg/kg, IV, q 12 h; n = 6) or firocoxib\textsuperscript{27} (0.09 mg/kg, IV, q 24 h; n = 6). For each horse, clinical blood samples were collected.
Determination of ex vivo COX activity

Ex vivo COX-1 activity was determined by measuring coagulation-induced TXB₂. Briefly, each residual whole blood sample was transported to the laboratory immediately after collection. Each sample was incubated at 37°C for 1 hour, after which it was centrifuged at 2,000 X g for 5 minutes. Then, 100 µL of serum was harvested and added to 400 µL of methanol; the solution was centrifuged at 6,000 X g for 10 minutes and then stored at –80°C until COX-1 and COX-2 activity were determined by means of a technique described elsewhere. Measurement of ex vivo COX-2 activity was performed with modification of a described technique. Briefly, heparinized blood (500 µL) was transferred immediately after collection into 1.5-mL polypropylene tubes, and LPS (Escherichia coli 0111:B4) in 0.1% bovine serum albumin in PBS solution was added to achieve a final concentration of 100 µg/mL. A second aliquot of blood was used as the negative (unstimulated) control sample. All experiments were performed in triplicate. Samples were incubated at 37°C for 24 hours. After incubation, samples were centrifuged at 2,000 X g for 5 minutes. Plasma was harvested, and 100 µL of plasma was added to 400 µL of methanol. The solution was centrifuged at 6,000 X g for 10 minutes; supernatant was harvested and stored at –80°C until the time of analysis to measure concentrations of PGE₂ and PGE metabolite.

A 50-µL aliquot of supernatant was diluted in 150 µL of buffer from a commercially available EIA kit. In addition, the amount of PGE metabolite in plasma obtained at each time point was measured as an indicator of COX-2 activity with a commercially available EIA kit as described elsewhere. Briefly, all unstable metabolites of PGE₂ were converted to stable 13,14-dihydro-15-keto-PGÄ₂ for quantification by means of a commercial ELISA kit performed in accordance with the manufacturer’s instructions.

Determination of ex vivo drug concentration

Aliquots of serum obtained at each time point were submitted to an external laboratory. Concentrations of flunixin meglumine and firocoxib were determined by use of HPLC.

Data analysis

The relative change in concentration of TXB₂, PGE₂, and PGE metabolite before NSAID administration (baseline) and 2 and 24 hours after NSAID administration was calculated. Effects of time and treatment on COX-1 (TXB₂) and COX-2 (PGE₂ and PGE metabolite) activity and drug metabolite concentrations were determined by means of the Mann-Whitney U test. Bonferroni corrections were used to account for multiple comparisons. Analysis was performed with the aid of commercially available software. The relationship between measured drug concentration and overall COX activity as well as COX activity at each time point was determined by Spearman rank order correlation analysis. On the basis of the drug concentrations obtained by use of HPLC, the predicted percentage inhibition of COX-1 activity was calculated from in vitro inhibition-concentration curves and compared with the ex vivo inhibition of COX-1 activity. Bland-Altman analysis was performed with the aid of commercially available software to determine the level of agreement between the in vitro and ex vivo methods of measuring inhibition of COX activity. For correlation and agreement analyses, values of P < 0.05 were considered significant.

Results

Measurement of in vitro inhibition of COX-1 activity

For flunixin meglumine, best fit (r² = 0.999) of the inhibition-concentration curve was obtained by use of a 4-point sigmoid function represented by the following equation:

\[
\text{Percentage inhibition}_{\text{flunixin}} = -11.4541 + (111.509/\left[1 + e^{-18.841/11.7442}\right])
\]

For firocoxib, the best fit (r² = 0.952) of the inhibition-concentration curve was obtained by use of a 3-point sigmoid function represented by the following equation:

\[
\text{Percentage inhibition}_{\text{firocoxib}} = 46.7088/\left[1 + e^{-14.4442/2.4425}\right]
\]

Ex vivo effects of flunixin meglumine and firocoxib

Horses in the flunixin meglumine group (3 Warmbloods and 3 Thoroughbreds [4 geldings, 1 mare, and 1 stallion]) had a median age of 8 years (range, 1 to 17 years) and median body weight of 588 kg (range, 350 to 656 kg). Four horses underwent elective soft tissue procedures (routine castration, surgical removal of a small sarcoma, laparoscopic ovariecotmy, and exploratory laparotomy), and 2 horses underwent elective orthopedic procedures (lateral plantar neurectomy and resection of a spinous process). Horses in the firocoxib group (2 Warmbloods, 2 Thoroughbreds, and 2 ponies [5 mares and 1 stallion]) had a median age of 4 years (range, 1 to 10 years) and median body weight of 538 kg (range, 323 to 651 kg). All 6 of these horses underwent elective orthopedic procedures (arthroscopic procedures). There was no significant difference in age or body weight of horses between the groups.
DETERMINATION OF EX VIVO COX ACTIVITY

Baseline (before NSAID administration) coagulation–induced TXB2 concentration did not differ significantly between the treatment groups. At 2 and 24 hours, coagulation-induced TXB2 concentration was significantly ($P = 0.03$) reduced, compared with the baseline value, in horses receiving flunixin meglumine only (Figure 1). At 2 and 24 hours, the relative coagulation-induced TXB2 concentration was significantly ($P = 0.005$) greater in horses receiving firocoxib, compared with that in horses receiving flunixin meglumine. There was no significant difference in baseline LPS-stimulated PGE2 or PGE metabolite concentrations between treatment groups. There was no significant difference in LPS-stimulated PGE2 or PGE metabolite concentrations between baseline and 2 or 24 hours in either treatment group. There was no significant difference in concentrations of LPS-stimulated PGE, (Figure 2) or PGE metabolite (Figure 3) between treatment groups.

DETERMINATION OF EX VIVO DRUG CONCENTRATIONS

Before NSAID administration, concentrations of flunixin meglumine and firocoxib were 0 µg/mL for all horses. Median flunixin meglumine concentration was 2.55 µg/mL (range, 2.45 to 3.82 µg/mL) at 2 hours and 1.29 µg/mL (range, 0.18 to 8.74 µg/mL) at 24 hours after administration. Median firocoxib concentration was 43.0 ng/mL (range, 30.8 to 78.0 ng/mL) at 2 hours and 35.4 ng/mL (range, 21.6 to 40.7 ng/mL) at 24 hours after administration.

Analysis of the combined time points revealed no significant correlation between flunixin meglumine or firocoxib concentration and relative or abso-
lute concentrations of TXB₂, PGE₂, or PGE metabolite. At 2 hours, the absolute concentrations of TXB₂ and PGE₂ were significantly correlated with the flunixin meglumine concentration ($r^2 = 0.889; P = 0.017$) and firocoxib concentration ($r^2 = 0.889; P = 0.017$). At 24 hours, the flunixin meglumine concentration was significantly correlated with the absolute concentration of TXB₂ ($r^2 = 0.784; P = 0.033$) and PGE metabolite ($r^2 = 0.707; P = 0.036$). In vitro and ex vivo inhibition of COX-1 activity differed significantly for both flunixin meglumine ($P < 0.001$; Figure 4) and firocoxib ($P = 0.040$; Figure 5).

Bland-Altman analysis revealed a mean difference between in vitro and ex vivo COX-1 activity. The mean
difference was 69.8% (95% CI, 37.0% to 102.6%) for flunixin meglumine (Figure 6) and -42.0% (95% CI, -181.5% to 97.6%) for firocoxib (Figure 7).

Discussion

The present study was conducted to determine the ex vivo effects of firocoxib and flunixin meglumine on COX activity in equine patients. Residual blood collected for clinical monitoring of patients undergoing elective surgery was used. Analysis of the results of this study suggested that firocoxib did not significantly inhibit COX-1 activity in clinical patients. This supports the findings of another study in which investigators found less inhibition of COX-1 activity by firocoxib than by flunixin meglumine in small intestinal ischemia-reperfusion injury. We did not identify a significant change in indicators of COX-2 activity, LPS-stimulated PGE2 concentration, and PGE metabolite concentration after surgery in either treatment group. Given that this was a clinical study, we did not include a negative control group that would have quantified the effect of surgery alone on COX-2 activity. However, our findings indicated that the effect of firocoxib on COX-2 activity was comparable to that of flunixin meglumine, and both appeared to prevent an increase in COX-2 activity in the 24 hours after surgery.

A limitation of the present study was the inability to match surgical procedures between groups, given that we used blood samples from clinical equine patients. All horses had no evidence of severe disease affecting >1 body system, and no horse included in the study had abnormal results for physical examination or hematologic evaluation. All procedures performed were considered elective. The commercial preparation of firocoxib is licensed in the United Kingdom for the alleviation of pain and inflammation associated with osteoarthritis and alleviation of lameness in horses. Because of the narrow spectrum of conditions licensed for treatment with firocoxib, only horses with evidence of joint disease undergoing arthroscopy were included in the firocoxib group. This procedure is minimally invasive and unlikely to induce extensive systemic inflammation. In comparison, the flunixin meglumine group included horses undergoing soft tissue procedures that could potentially have resulted in greater COX stimulation than for arthroscopy. However, there was no significant difference in preoperative prostanoid concentrations between groups, and postoperative prostanoid concentrations were not increased in the flunixin meglumine group. A horse in the flunixin meglumine group underwent exploratory laparotomy to evaluate a hepatic mass that was confirmed to be a hydatid cyst, which likely constituted an incidental finding. A study conducted to examine the effect of surgery on the early (<24 hours) inflammatory response found a significant difference in serum amyloid A concentrations between procedures with minimal tissue injury (eg, arthroscopies) and procedures with intermediate tissue injury (eg, laryngeal surgeries and castrations), but no significant difference in serum amyloid A concentrations was found between surgeries involving minimal and major tissue injury. In addition, investigators in that same study found no significant effect of tissue injury on other variables of inflammation, including WBC count and serum iron concentration. These findings suggest that although there may be a difference in the degree of inflammation stimulated by surgery, it is minimal during the first 24 hours after surgery. In the study reported here, horses had no abnormal physical examination findings or results for hematologic evaluation before surgery and did not develop complications after surgery. In light of these findings and the limitations imposed by the clinical study design, enrolling patients undergoing procedures that involved mild to moderate tissue damage was considered acceptable. The findings of this study supported the hypothesis that firocoxib has ex vivo COX-1-sparing effects; however, further studies with more homogeneous groups would yield more conclusive evidence.

To our knowledge, the present study was the first that has been conducted to examine the correlation between serum NSAID concentration and COX activity in horses. Significant correlations between indicators of COX activity and flunixin meglumine concentration were identified at 2 and 24 hours after NSAID administration and between indicators of COX activity and firocoxib concentration at 2 hours after NSAID administration. Lack of a consistent correlation between NSAID concentration and inhibition of COX activity was likely the result of multiple factors, including a short metabolite half-life, differences in drug half-life between groups, differences among individual patients, and the use of small heterogeneous groups. Group size was determined on the basis of the predicted difference in the drugs selectivity for COX. Available in vitro data indicate an approximately 265-fold difference in selectivity between firocoxib and nonselective COX inhibitors. Power analysis determined a minimum group size of 2 horses to yield a power of at least 0.80, assuming a high SD of 130 between samples. However, it was expected that in vitro selectivity data might be overestimating the selectivity of firocoxib, and each group in the present study included 6 horses, which should have allowed detection of other significant relationships between drug and metabolite concentrations and enzyme activity, disease state, and age or breed differences. Further studies are warranted.

Drug concentrations in the present study were in the range expected on the basis of pharmacokinetic parameters reported for these drugs. Pharmacokinetic studies are performed on healthy animals, and the applicability of these parameters to clinical patients is debatable. Patients often have serious chronic or acute disease or receive multiple drugs (eg, anesthetics, antimicrobials, or prokinetics) simultaneously. In the study reported here, all horses received antimicrobials perioperatively and anesthetics (general anesthesia). These could potentially have interfered with

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metabolism and efficacy of the drugs of interest. No difference was expected between the predicted drug concentrations calculated from the pharmacokinetic parameters and the actual drug concentrations, considering that these patients had no abnormal findings for physical examination or results for hematologic evaluation. This would suggest that pharmacokinetic parameters for these NSAIDs determined in experimental animals are applicable to clinical patients undergoing elective surgery.

Studies conducted to examine the inhibitory effects of NSAIDs on COX in horses are based on in vitro methods. In the present study, we examined the ability of in vitro methods to predict ex vivo inhibition of COX-1 activity by flunixin meglumine and firocoxib in horses. The function that best fits the concentration-inhibition curve in vitro was used to predict the relative inhibition expected for the actual drug concentrations measured by use of HPLC in the samples obtained ex vivo. Results of Bland-Altman analysis revealed a high bias, particularly for flunixin meglumine (95% CIs for flunixin meglumine were both > 0 and indicated that ex vivo inhibition of COX activity was more efficient than in vitro inhibition), which suggested very poor agreement between the ex vivo and in vivo methods. The wide 95% CIs (> 100%) for firocoxib suggested that estimation of ex vivo inhibition of COX-1 activity by use of in vitro data was highly variable. Other authors have described the manner by which in vitro data on inhibition of COX activity in humans differs between NSAIDs. Investigators of that study found that the overall value of in vitro assays to predict ex vivo inhibition of COX activity differed among drugs: the ex vivo effect of diclofenac was reliably predicted in vitro, whereas ex vivo and in vitro inhibition differed significantly for ibuprofen on COX-2 activity and meloxicam on COX-1 activity. Investigators of another study found that etodolac was preferentially selective for COX-2 in vitro but not ex vivo in horses. In veterinary species, studies have detected significant differences in the in vitro and ex vivo COX selectivity ratio (concentration required to inhibit enzyme activity by 50%) of NSAIDs. Calculation of the in vitro COX selectivity ratio has been reported previously and was not an objective of the present study; calculation of an ex vivo COX selectivity ratio is not possible in clinical patients. The significant difference between calculated and actual measurements in COX-1 activity further supports the need to assess NSAID efficacy ex vivo as well as in vitro in horses. However, it is also possible that variability in procedures performed in the flunixin group could have caused variable degrees of inflammation and might have contributed to the bias to some extent. In addition, the in vitro and ex vivo experiments were conducted in separate populations of horses. However, we believe that the comparison of in vitro and ex vivo methods warrants further investigation and comparison for COX-2.

The present study was not designed to compare analgesic effects of these drugs. Despite the short half-life of flunixin meglumine (2.1 to 4.2 hours), this drug can significantly reduce tissue production of PGs in exudates for up to 24 hours, which is well after the elimination of flunixin meglumine from the bloodstream. The persistence of firocoxib at a peripheral site of inflammation remains undetermined; however, the long half-life (29 to 31 hours) after once-daily administration should guarantee persistence of the inhibition of COX activity in peripheral tissues throughout the course of treatment. Evidence suggests that firocoxib is effective for controlling orthopedic pain in both experimental and clinical settings. None of the clinical patients in the firocoxib group required further administration of analgesic by attending clinicians, who were not involved in the study, during the first 24 hours after surgery (ie, after a single dose of firocoxib). The author’s subjective clinical impression was that firocoxib might offer adequate analgesia for minimally invasive elective surgical procedures such as arthroscopy in horses, but further studies would be required to enable objective assessment of the efficacy of firocoxib as a perioperative analgesic. Results for experiments in 1 study revealed that firocoxib was as effective as flunixin meglumine for managing signs of pain in healthy horses undergoing ventral midline celiotomy and jejunal ischemia-reperfusion injury without enterectomy. In that study, firocoxib was also as effective as flunixin meglumine at inhibiting PG production driven by ischemia-reperfusion injury. Whether firocoxib would control inflammation and the associated pain from more severe naturally occurring disease or colic remains undetermined and should be evaluated in future studies.

Firocoxib did not inhibit ex vivo COX-1 activity in equine patients that underwent elective surgery and could potentially offer an alternative for the treatment of pain and inflammation in patients for which the use of nonselective NSAIDs is contraindicated. Further studies are required to evaluate the use of firocoxib in patients with clinical conditions for which its use is not currently licensed, including intestinal ischemia, right dorsal colitis, or prerenal or intrinsic acute renal failure, before it can be recommended for use in these conditions.

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Footnotes
e. Prostaglandin E2 EIA kit—monoclonal, Cayman Chemical Europe, Tallinn, Estonia.
f. Prostaglandin E metabolite EIA kit—monoclonal, Cayman Chemical Europe, Tallinn, Estonia.
g. UNIRELAB srl, Unipersonale, Rome, Italy.
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